COBISS: 1.08
Agris category code: L51

SINGLE AND COMBINED EFFECT OF DIETARY THYME (*Thymus vulgaris*) AND SPIRULINA (*Arthrospira platensis*) ON BACTERIAL COMMUNITY IN THE CAECUM AND CAECAL FERMENTATION OF RABBITS

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ABSTRACT

The objective of this study was to evaluate the effect of supplementation of the growing rabbits' diet by Spirulina or/and Thyme (food supplement) on the composition and amount of the microbiota and production of volatile fatty acids of the rabbits' caecum. We used classical culturing methods and after bacterial DNA extraction, the quantity of bacteria (belonging to phylum Firmicutes and Bacteroidetes) were determined by qPCR reactions with the aid of bacterial ribosome coding DNA at the Molecular Biology Laboratory, University of Kaposvár. The experiment was carried out using rabbits (Pannon White) from rabbit-farm of Kaposvar University. Young rabbits were weaned on the 35th day after birth. All experimental animals were the same age. Duration of Spirulina and/or Thyme supplementation –after weaning- was 48 days. Samples were collected on the 14th, 28th and 48th day of the supplementation. No dietary effect on pH of the caecal content was detected. The number of *Escherichia coli*, total anaerobic and strictly anaerobic bacteria decreased by age, no effect of the diet could be demonstrated. Supplementation with Thyme resulted in slightly higher ratio of propionic acid, but the difference was not significant. In conclusion, Spirulina and/or Thyme supplementation of diet after weaning had no substantial effect on the volatile fatty acid (VFA) production, while the classical microbiological determination did not find a substantial effect on the composition of the caecal microbiota. By the Quantitative PCR method we measured significantly lower bacterial copy numbers in the samples of ST treated group. This indicates that the ST (Spirulina 5% and 3% Thyme) feed supplementation has an antimicrobial effect on the investigated bacterial groups in the caecum of growing rabbit.

Key words: rabbits / animal nutrition / Spirulina / Thyme / microbiology/ caecal microbiota / volatile fatty acids

1 INTRODUCTION

The composition and the activity of the caecal microbiota have a strong influence on health, because of their role in nutrition, pathogenesis and immune function (Gibson and Roberfroid, 1995). In addition to classical culturing methods, molecular microbiology techniques have been recently introduced for determination of changes in the microbiota. In connection with the ban of using antibiotics as growth promoters in the EU several studies have been carried out on different feed additives – for example herbal extracts – as alternatives for antibiotics, because of their growth-promoting effects. Thyme has been reported for its antimicrobial and antioxidant properties (Dorman and Deans, 2000). Spirulina is a type of blue-green algae that is rich in protein, vitamins, minerals, and carotenoids. It has been used as human food supplement for over 20 years, because of its high nutrient content, including B complex vitamins, beta-carotene, vitamin E, manganese, zinc, copper, iron, selenium, and gamma linolenic acid (Belay *et al.*, 1996). Several studies have been shown that Spirulina has beneficial biological activities, such as immunomodulation, antioxidant, anticancer, antimicrobial and probiotic effects (Belay, 2002).

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The objective of this study was to evaluate the effect of supplementation (between the ages of 5–11 weeks) of the growing rabbits' diet by Thyme and/or Spirulina on the bacterial community and fermentation in the rabbits' caecum.

2 MATERIALS AND METHODS

2.1 ANIMALS AND EXPERIMENTAL DESIGN

Rabbits involved in the experiment received the control pellet (C) from the age of 3 weeks. After weaning (at the age of 5 weeks) the rabbits were housed in wire net cages (0.61 × 0.32 m, 3 rabbits/cage). The temperature and daily lighting in the house were 16–18 °C and 16 hours, respectively. The weaned rabbits were randomly sorted to 4 groups (42 rabbits/group). Rabbits of the control group (C) received a pellet without any supplementation throughout the experiment (up to11 weeks of age). In the other groups the pellet was completed by 5% Spirulina (S), or 3% Thyme (T) or by both (ST) for the whole (5–11 week) growing period. More details about housing and feeding conditions are described in Gerencsér *et al.* (2012).

2.2 SAMPLE COLLECTION

During the treatment at 14, 28 and 48 days (sampling points: 1, 2, 3, respectively) 6 healthy animals from each group (one animal/cage) were randomly selected and slaughtered at 02:00 p.m. The digestive tract was removed immediately and the caecum was separated. The quantity of the fresh caecal content was measured and pH value was determined. For microbiological determination, 1 g of the fresh caecal content was homogenised at room temperature and serial dilution was made with sterile isotonic solution under a carbon dioxide stream. For molecular tools and chemical analysis, some of the fresh caecal contents was frozen and stored at −20 or −80 °C until processing.

2.3 MICROBIOLOGICAL DETERMINATION

Total aerobic bacteria and total anaerobic bacteria were cultured on blood agar, prepared with 5% defibrinated calf blood. Samples were incubated at 37 °C for 24 h and 72 h, under aerobic and anaerobic condition, respectively. Coliforms and *Escherichia coli* sub-spp. were cultured on a Chromocult differentiation medium (Merck, Darmstadt, Germany). Samples were incubated at 37 °C, under aerobic conditions, for 24 h. *Campylobacter* spp. was cultured on blood-free selective agar (Merck). Samples were incubated at 37 °C, under aerobic conditions, for 48 h. The obligate anaerobe organisms were cultured on Schaedler's agar (Scharlan Chemie, Barcelona, Spain), the selectivity of which was increased by the addition of esculin, neomycin (Merck) and Ferri-ammonium-citrate (Scharlan Chemie, Barcelona, Spain). Petri dishes were placed into Anaerocult culture dishes (Merck), in which the anaerobic conditions were ensured with the help of an Anaerocult A (Merck) gasifying bag. The samples were incubated 37 °C for 96 h. After the incubation had elapsed, the colonies were counted (ISO 4833:2003) with Acolyte colony counter (Aqua-Terra Lab, Veszprem, Hungary). The colony counts were expressed in log10 colony forming units (CFU) related to 1 g of sample.

2.4 DETERMINATION OF VOLATILE FATTY ACIDS (VFA) CONCENTRATION

VFA concentrations of 11 week old growing rabbits' caecal samples were measured. Approximately 5 g digesta per rabbit were stored in fridge (−20 °C) until measurement. The concentration of VFA was measured by gas chromatography (Shimadzu GC 2010, Japan), using external standard.

2.5 DNA EXTRACTION AND QPCR

Total DNA from about 200 mg of caecal sample of ST treated animals was extracted and purified using the QIAamp ® DNA Stool Mini Kit (50) (QIAGEN) according to the manufacturer's instructions. DNA concentrations were measured using Smart Spec Plus Spectrophotometer (BioRad). The concentrations of all DNA samples were set to 60 ng/µl. After the preparation of caecal samples (bacterial DNA extraction) the quantity of bacteria (belonging to phylum Firmicutes and Bacteroidetes) were determined by QPCR reactions. QPCR was carried out in a 25 µl/tube reaction mixture containing 12.5 µl Brillant II SYBR QPCR Low Rox Master Mix (Agilent Technologies), 0.2 µM of each primer (Table 1.), 10.5 µl sterile distilled water and 1 µl of DNA extract (60 ng/µl). Sample measuring robot (QIAgility, QIAGEN) was used to fill 96-well plates with PCR reaction components and samples, which – compared to manual measurement – reduced the standard deviations. The PCR program consisted of 10 min at 95 °C, 40 cycles with 30 sec at 95 °C, 1 min at 60 °C.

MxPro 3000P QPCR apparatus (Agilent Technologies) was used for the bacterial target sequence ampli-

Table 1: *Oligonucleotide sequences used for QPCR*

Group	Item	Oligonucleotide sequence (5'–3')	T_a * (°C)	References
Clostridium coccoides	Forward (Cc1)	GAC GCC GCG TGA AGG A	60	Firmesse *et al.* (2008)
	Reverse (Cc2)	AGC CCC AGC CTT TCA CAT C		
Clostridium leptum	Forward (Cl9)	CCT TCC GTG CCG SAG TTA	60	Firmesse *et al.* (2008)
	Reverse (Cl8)	GAA TTA AAC CAC ATA CTC CAC TGC TT		
Bacteroides	Forward (Bs2)	CCT WCG ATG GAT AGG GGT T	60	Firmesse *et al.* (2008)
	Reverse (Bs1)	CAC GCT ACT TGG CTG GTT CAG		

*T_a – Annealing temperature

fication applying primers and SYBR Green in the experimental assembly. Specificity of PCR reactions were checked by melting point analysis. All samples were measured in three technical triplicates. C_t values of the samples – having equilibrated concentrations – were the basis of monitoring the changes of bacterial community. After cloning of the amplified PCR products (external lab orders), we determined the plasmid concentrations, and dilution series were prepared (standard curve). The bacterial contents of samples were calculated with the aid of that. The obtained copy numbers of the samples were adjusted to one gram of caecal content.

The bacterial quantification based on real-time PCR. Real-time PCR has been developed for monitoring the amplification reaction. SYBR Green I is a double-stranded DNA binding dye that allows the detection of PCR products, including the DNA extracted from bacterial samples. The bacterial quantification data using real-time PCR is commonly expressed as absolute quantities in units such as copies/g, colony-forming unit (CFU)/mL or Log CFU/g of samples such as digesta. In Quantitative PCR/qPCR gives realtime monitoring, each cycle is detected via light (monochromatic or narrow wavelength range) exitation of a dsDNA binding fluorescent dye (SYBR Green): the intensity of the emitted light correlates with the amount of product generated by PCR, allowing quantitative detection (Navidshad *et al.* 2012).

2.6 DATA ANALYSIS

Data from classical microbiological determination and gas chromatography were analysed by using GLM (General Linear Model) procedure of SPSS (2002), version 10.0. Group of diet and age were factors. The formula of General Linear Model included the following:

$$y_{ij} = \mu + \text{sampling point}_i + \text{diet}_j + \text{sampling point}_i \times \text{diet}_j + e_{ij}$$

where y is the measured pH values, etc. (see Table 2., column 1), μ is the general mean, sampling point is the

Table 2: *Effect of different pellets inclusion on caecal digesta traits of growing rabbits*

Traits	Experimental diets				Sampling point (d)				P-value		
	C	S	T	ST	1	2	3				
N	18	18	18	18	24	24	24	RSD	Diet	Sampling point	Diet × Sampling point
pH of caecal cont.	6.37	6.46	6.44	6.49	6.35	6.50	6.47	0.23	0.501	0.122	0.830
Total aerobic bacteria [1]	6.91	6.87	7.02	6.53	7.02	6.91	6.57	0.71	0.128	0.055	0.112
E. coli spp. [1]	3.95	3.92	3.70	3.50	3.91[b]	4.19[b]	3.21[a]	0.84	0.225	0.000	0.337
Campylobacter spp. [1]	3.81	4.01	3.59	3.51	3.80	3.66	3.01	0.86	0.536	0.577	0.925
Total anaerobic bacteria[1]	8.61	8.52	8.54	8.39	8.82[b]	8.31[a]	8.39[a]	0.43	0.357	0.000	0.093
Strictly anaerobic bacteria[1]	7.95	7.86	8.00	7.92	8.05[b]	8.00[b]	7.75[a]	0.35	0.642	0.005	0.457

[1] Germ counts expressed in log10 CFU/g caecal digesta. Different superscripts mean significant differences between ages.

Table 3: *Effect of different pellets inclusion on VFA content of the caecal digesta at 77 days of age, n = 6*

	Experimental diets								
Traits	C	Sd	S	sd	T	sd	ST	sd	P-value
Dry matter content (w/w%)	22.6	1.12	22.5	1.60	22.6	0.39	22.5	0.87	0.990
Total VFA content (mmol/kg)	35.1	5.40	31.0	9.58	34.3	6.40	36.1	10.0	0.715
Acetic acid %[1]	74.8	1.78	73.6	1.60	74.1	1.12	73.9	1.17	0.516
Propionic acid %[1]	6.97	0.53	7.19	1.26	8.46	1.33	7.83	0.93	0.095
Butyric acid %[1]	18.2	1.75	19.3	2.17	18.8	4.29	17.3	2.12	0.659

[1] Proportion within total VFA content

event of caecal content sampling (1, 2, 3), indicates the impact of the combined supplemented diet and e is the residual error.

VFA content of 11th week old rabbits' caecal digesta was analysed by using one way ANOVA. The significance of differences was tested by Tukey post hoc test.

The copy numbers of the investigated bacterial groups were visualised in copy numbers vs. sampling-events coordinates (data not shown). The qPCR dataset was analyzed with the GLM to determine differences between the amount of investigated bacterial groups, where the sampling points and ST (Spirulina 5% and Thyme 3%) supplemented diet were included as fixed effects and bacterial copy numbers as dependent variable. The formula of General Linear Model included the following:

$$y_{ij} = \mu + \text{sampling point}_i + \text{diet}_j + \text{sampling point}_i \times \text{diet}_j + e_{ij}$$

where y is the copy number of the investigated bacteria (e.g. Bacteroides), μ is the general mean, sampling point is the event of caecal content sampling (1, 2, 3), indicates the impact of the combined supplemented diet and e is the residual error. The significance of differences was tested by Tukey post hoc test.

3 RESULTS AND DISCUSSION

No effect of the diet on the pH of caecal content was detected (Table 2). The number of *E. coli*, total anaerobic and strictly anaerobic bacteria decreased by age, and no effect of the diet could be demonstrated. Supplementation with Thyme resulted in a slightly higher ratio of propionic acid, but the difference was not significant (Table 3).

When using the molecular genetics tool, qPCR, all investigated bacterial group displayed the similar, increasing trend in the control and ST treated group (Table 4.). The amount of *Bacteroides*, *Clostridium leptum* and *Clostridium coccoides* in 1 g caecal content of the ST treated samples were significantly less ($P < 0.05$) (Table 5.) than that of the control group at each sampling point.

However the classical microbiological determination was able to demonstrate significant differences between the sampling points in case of *E. coli*, total anaerobic and strictly anaerobic bacteria (suggesting declined bacterial load with age, Table 2.), this significance clearly disappears when the effect of the investigated diet -as an interaction with sampling points- was included into the calculations. As a consequence we cannot state any substantial effect of supplementing the diet by Spirulina and/or Thyme, after weaning on the composition of the caecal microbiota based on classical microbiological approach.

By Quantitative PCR method we measured increasing copy numbers of bacteria with age both in the con-

Table 4: *Average copy number of 1 gram caecum samples at different sampling points and their deviations*

Sampling point – day of the treatment	Number of copies											
	Bacteroides				*Clostridium leptum*				*Clostridium coccoides*			
	C	D	ST	D	C	D	ST	D	C	D	ST	D
1 (14)	97034	2022	91530	910	95707	2082	89717	831	110437	2609	102985	1011
2 (28)	97052	2539	97683	2361	96974	5399	95603	2487	109212	7812	109837	2739
3 (48)	99275	504	99028	1715	99018	2895	96673	1707	114646	3494	111055	2008

C – Control, D – Deviation, ST – Spirulina and Thymus combined diet

Table 5: *Differences between the Control (C) and treated (ST) groups, results of the Tukey post-hoc test*

	Mean diff.	Standard error	P-value
Bacteroides C-ST	2596	718	0.005
Clostridium coccoides C-ST	4950	1435	0.007
Clostridium leptum C-ST	3663	1229	0.020

trol (C) and in the SP diet (Spirulina 5% and 3% Thyme) diet. Comparing copy number values of SP diet to that of the corresponding control groups, antimicrobial effects ($P < 0.05$) were demonstrated on the investigated bacterial groups in the caecum.

The reason of this difference from the classical microbiological approach may come from the sensitivity of molecular microbiology techniques (Tannock *et al.*, 2000; Takahiro *et al.*, 2003). In case of Firmicutes (*Clostridium leptum, Clostridium coccoides*) these observed trends are in good accordance to the findings in humans and rabbit, such as body weight (Turnbaugh *et al.*, 2009) and age (Combes *et al.*, 2011) and are associated with a larger proportion of Firmicutes.

In the present study, the dietary change modifies the investigated bacterial groups, but the quantity of total bacteria has not been measured by qPCR. The decrease of proportion of some species as a consequence of a change in substrate availability could be compensated for by the increase of proportion of other groups better adapted to the available nutrients (Zoetendal *et al.,* 2004). The consumption of resources that govern ecosystem processes through two types of effects: a `complementarity effect', which occurs through either resource partitioning or facilitative interactions between species and a `selection effect', which occurs whenever species diversity is correlated with the chance of resource use (Cardinale *et al.*, 2002).The trend is valid for each test group, not the total number of bacteria.

To have further insight into how Thymus and Spirulina affect the microbiota, collection of more samples from different animals are being done and other experimental/analytical setups being considered.

4 ACKNOWLEDGEMENT

Research was funded by Padova University research funds (Progetti di Ricerca di Ateneo 2011) code: CPDA117509/11, and the EU project TÁMOP 4.2.1.B-10/2/KONV-2010-0002.

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